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Research article

DETECTION OF *icaA* AND *icaD* GENES OF *Staphylococcus aureus* ISOLATED IN CASES OF BOVINE MASTITIS IN THE REPUBLIC OF SERBIA

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Staphylococcus aureus (*S. aureus*) is the most common agent of contagious mastitis that causes serious health and economic problems. The ability to form biofilms is an important virulence factor of *S. aureus* for the establishment of persistent infections. This study is aimed to investigate the presence of *icaA* and *icaD,* two genes of importance for the biofilm formation in *S. aureus* bovine mastitis isolates. In order to isolate and identify *S. aureus*, 1555 milk samples were collected from 401 cows, located in different regions of the Republic of Serbia. Using the conventional microbiological methods 100 isolates were characterized as coagulase-positive staphylococci. After primary biochemical identification, the species confirmation of 44 *S. aureus* isolates was done using MALDI-TOF Mass Spectrometry and PCR technique, targeting the *S. aureus*-specific *nuc* gene. Among all investigated *S. aureus* isolates 25.0% harbored both *icaA* gene and *icaD* genes. The presence of *icaD* gene alone was confirmed in 40.9% of cases totaling *icaD* positive isolates to 65.9%. The remaining 34.1% of *S. aureus* isolates were negative for the presence of both genes. The results of the present study indicate the existence of potential biofilm-producer strains in different regions of the Republic of Serbia, both under intensive and semi-extensive cows breeding.

Key Words: biofilm, *icaA* gene, *icaD* gene, mastitis, *Staphylococcus aureus*

INTRODUCTION

Mastitis is the most important disease in dairy cows leading to substantial economic losses in the primary milk production worldwide [1-2]. *S. aureus* has been described as a major pathogen responsible for this disease among more than 137 different microorganisms associated with the etiology of mastitis [3,4]. The infected mammary gland is the main reservoir of *S. aureus* while the transmission between cows usually occurs during milking [5]. Early detection and characterization of *S. aureus* strains are very important for mastitis prevention and management [6]. In veterinary microbiology,

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several techniques are used for the identification and the characterization of *S. aureus* mastitis isolates. The routine microbiological methods applied to microorganism identification require a lot of time and use of various biochemical tests, as well as the evaluation of experts [7]. Compared to the conventional phenotypic methods, molecular methods provide faster and more accurate responses during the identification of the microorganisms [8,9]. MALDI-TOF Mass Spectrometry and PCR are valuable in the rapid identification of this contagious mastitis agent while the PCR technique is also used to test the pathogenic potential of *S. aureus* mastitis isolates.

Many virulence factors are involved in *S. aureus* confrontation of the mammary gland defense system. The ability to form biofilm is an important virulence factor of *S. aureus* in the pathogenesis of mastitis [10,11]. Biofilm formation is a very complex process that begins by adhesion of *S. aureus* to the tissue surface, using surface-anchored proteins which bind to host matrix proteins (MSCRAMMs), exposed due to tissue damage [12,13]. After primary adhesion, bacterial cells multiply and produce an extracellular biofilm matrix, also known as slime, which provides interactions between bacterial cells. The main component of the *S. aureus* slime is the exopolysaccharide poly-N-acetyl-β-1,6-glucosamine (PNAG), synthesized by enzymes encoded in the intercellular adhesion (*ica*) locus [14]. The *ica* locus belongs to the "accessory genes" of the genome [15] and contains *icaA, icaB, icaC* and *icaD* genes, of which *icaA* and *icaD* play a significant role in *S. aureus* biofilm formation [16]. The *icaA* gene encodes enzyme N-acetylglucosaminyl transferase whose enzymic activity becomes significant only in the case of *icaD* gene expression [15]. The product of *icaC* gene is involved in the translocation of the poly-N-acetylglucosamine molecule to the surface of the bacterial cell while *icaB* gene encodes the surface-attached protein which performs the deacetylation reaction essential for the interactions between bacterial cells and promotes biofilm development [15,17].

Detection of the *icaA* and *icaD* genes using PCR assay is reliable for determining the potential of *S. aureus* isolates to produce biofilms and may help in the rapid detection of biofilm-producer strains [6]. Therefore, the aim of this study was to investigate the frequency of the biofilm producing related genes, *icaA* and *icaD,* occurrence in *S. aureus* mastitis isolates originated from different regions of the Republic of Serbia.

MATERIALS AND METHODS

Milk sampling

A total of 1555 milk samples were collected from 401 cows from different regions of the Republic of Serbia. Milk samples were collected from udder quarters with increased somatic cell counts, as determined by the California mastitis test or from cows with clinical mastitis. Before milking the udder skin was cleaned, washed and dried. A volume of 10 ml of milk per udder quarter was collected in sterile properly labeled tubes, after disinfection of teat ends with 70% ethanol. Milk samples were kept

at 4°C and transported to the laboratory of the Department for Food Hygiene and Technology of Animal Origin, Faculty of Veterinary Medicine, University of Belgrade.

Microbiological assessment

The isolation of *S. aureus* was carried by streaking 0,1 mL of each milk sample, respectively, onto the Columbia agar plates supplemented with 5% sheep blood followed by 24h incubation at 37°C under aerobic conditions. After incubation, typical colonies were subjected to Gram-staining and positive isolates subcultured on Trypticase soy agar 24h at 37°C for catalase and coagulase testing. API Staph-Ident system (BioMérieux, France) was used for biochemical identification of coagulase-positive staphylococci. 44 selected *S. aureus* isolates were then subjected to the species confirmation using *MALDI-TOF Mass Spectrometry* and *PCR*.

MALDI-TOF Mass Spectrometry

The species confirmation of 44 isolates was done using Vitek MS (bioMérieux, France) based on *MALDI-TOF Mass Spectrometry* technology. Preparation of investigated isolates was done according to the manufacturer. *Escherichia coli* ATCC® 8739 was used for calibration of the apparatus while *VITEK MS V2.0 Knowledge Base – Industry Use* was used for results reading.

DNA extraction

Extraction of genomic DNA was carried out by the boiling method. *S. aureus* isolates were subcultured on Trypticase soy agar at 37°C for 24h. After the incubation period, a fresh colony of each isolate was suspended in 500 µL of DNase-RNase free water. The suspension was held in a water bath at 100ºC for 10 min and then cooled on over the next 10 min. After centrifugation at 10 000 rpm for 5 min, the supernatant containing bacterial DNA was used as a template for PCR amplification.

PCR assays

The molecular identification (species confirmation) of 44 chosen isolates was done using PCR targeting S. *aureus*-specific *nuc* gene (encoding thermostable nuclease). Primers used for the amplification of the *nuc* gene are shown in Table 1. All PCR reactions were performed in 50 µL reaction volumes containing 25 µL Dream Taq Master Mix (2X) that contains 2× Dream Taq buffer, 4 mM $MgCl_2$ and 0,4 mM of each of the 4 dNTPs (Thermo Scientific, Lithuania), 1 µL of each primer, 1µL of template DNA and nuclease free water to 50 µL. DNA amplification was performed in a FlexCycler (AnalyticJena, Germany). The program for amplification of *nuc* gene was: (1) initial denaturing step at 95°C for 5 min; (2) 30 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C; and (3) final extension step at 72°C for 7 min. Amplification products were analyzed by electrophoresis on 1.5% (wt/vol) agarose (TopVision

agarose Thermo Scientific, Lithuania) gel, after staining with ethidium bromide. *S. aureus* ATCC 25923 was used as a positive control while Gene Ruler 100 bp DNA ladder (Thermo Fisher Scientific, Lithuania) was used as the molecular weight marker.

In order to determine the potential of *S. aureus* isolates to produce slime, PCR assay targeting *icaA* and *icaD* genes was performed [13]. Primers used for the amplification of *icaA* and *icaD* genes are shown in Table 1. All primers used in this study were obtained from Invitrogen (United States). Ten microliters of the extracted DNA was used as a template in a 50 μL PCR mixture, that contained 25 μL Dream Taq Master Mix (2X) (Thermo Scientific, Lithuania), 5 μL of each primer, and nuclease free water to 50 μL. The amplification of *icaA* and *icaD* gene was performed as follows: (1) initial denaturing step at 92ºC for 5 min; (2) 30 cycles of 92ºC for 1 min, 49ºC for 50 s and 72ºC for 1 min; and (3) final extension step at 72ºC for 7 min. *S. aureus* ATCC 6538 was used as a positive control for both genes [21] and Gene Ruler 100 bp DNA ladder as the molecular weight marker. The PCR for *icaA* and *icaD* genes have amplified the products of 1315 bp and 381 bp, respectively (Figure 1). Electrophoresis was performed in 1.5% (wt/vol) agarose gel stained with ethidium bromide.

Table 1. Primers used for the amplification of *nuc*, *icaA* and *icaD* genes

Gen	Primer	Sequence (5'-3')	Reference
Nuc	nuc - f	TCA GCA AAT GCA TCA CAA ACA G	[8]
	nuc - r	CGT AAA TGC ACT TGC TTC AGG	
IcaA	icaA - f	CCT AAC TAA CGA AAG GTA G	[13, 17]
	icaA - r	AAG ATA TAG CGA TAA GTG C	
IcaD	icaD - f	AAA CGT AAG AGA GGT GG	[13, 17]
	icaD - r	GGC AAT ATG ATC AAG ATA C	

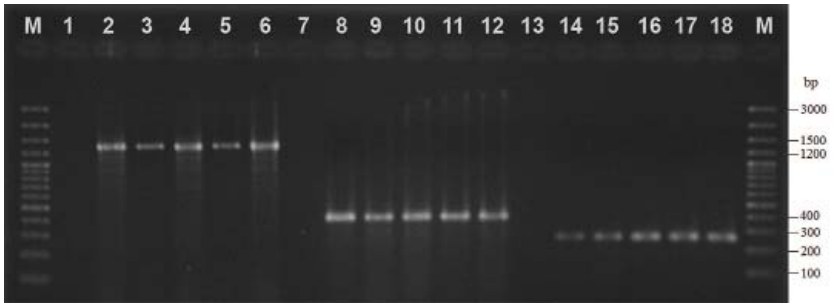

Figure 1. Agarose gel electrophoresis with PCR amplicons of *icaA*, *icaD* and *nuc* gene
Legend: M: molecular weight marker; 1: negative control for *icaA* gene; 2-5: *S. aureus icaA* (1315 bp) positive isolates; 6: positive control for *icaA* gene (*S. aureus* ATCC 6538); 7: negative control for *icaD* gene; 8-11 *S. aureus icaD* (381 bp) positive isolates; 12: positive control for *icaD* gene (*S. aureus* ATCC 6538); 13: negative control for *nuc* gene; 14-17: *S. aureus* isolates - species confirmation based on *nuc* gene (255 bp); 18: positive control for *nuc* gene (*S. aureus* ATCC 25923).

RESULTS

After conventional microbiological testing, 100 isolates of coagulase-positive staphylococci were isolated from 1555 milk samples. Further biochemical and molecular identification confirmed 44 selected isolates as *S. aureus* with the presence of species specific *nuc* gene in all examined isolates (Figure 1 and data not shown).

Among all investigated *S. aureus* isolates, 11 out of 44 (25.0%) harbored both *icaA* and *icaD* genes. The presence of *icaD* gene alone was observed in 18 out of 44 (40.9 %) *S. aureus*,isolates, concluding the total number of isolates with *icaD* gene to 29 (65.9%). The remaining 15 (34.1%) *S. aureus* isolates did not harbor any of the tested genes (Figure 2).

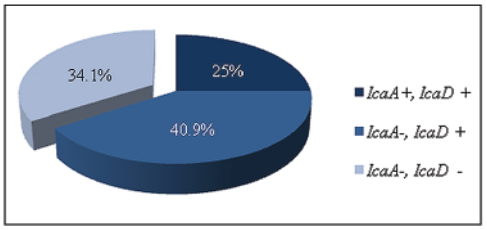

Figure 2. Results of *icaA* and *icaD* genes detection in *S. aureus* mastitis isolates

DISCUSSION

Due to the various virulence factors, *S. aureus* successfully opposes the mammary gland defense system leading to the development of the pathological process. The ability of *S. aureus* to form biofilm is an important factor in the mastitis pathogenesis that enables the long-term persistence of bacteria in the mammary glands, leading to the chronic form of the disease [10,19]. This ability is often associated with the decreased efficacy of antibiotic therapy and problems with infection eradication [20]. Rapid detection of biofilm producing *S. aureus* strains along with the knowledge of biofilm formation mechanisms enables early application of the proper corrective measures [21]. The most studied biofilm mechanism in *S. aureus* is *ica* operon dependent mechanism and previous research demonstrated that the majority of clinical isolates, both from human and bovine mastitis contain the *ica* operon [22]. Therefore, PCR assay standardized for detection of the *icaA* and *icaD* genes can determine the potential of *S. aureus* isolates to produce biofilm and may help in the rapid detection of biofilm-producer strains [6]. Among 44 confirmed *S. aureus* isolates investigated in this study, only 11 (25.4%) were positive for both *icaA* and *icaD* gene. Similar results were reported by Ciftci et al. [23] who determined the presence of both *icaA* and *icaD* gene in 25% of *S. aureus* mastitis isolates while Dhanawade et al. [24] have found 35.29% of *S. aureus* isolates positive for the presence of both genes. In contrast to our findings, Vasudevan et al. [10] and Fabres-Klein et al. [25] have found that all investigated mastitis isolates of *S. aureus* were positive for both genes. However, 18 of 44 (40.9%) isolates in the present study

were positive only for *icaD* gene. Arciola et al. [26] were not detected a gene deletion within the *ica* locus and pointed out that the genes of the *ica* locus are strictly linked to each other, so they are either all present or all absent. The findings of Ciftci et al. [23] speak in favor of results from this study, giving that they also determined only the presence of *icaD* gene in a portion of *S. aureus* mastitis isolates. The absence of *icaA* gene detected in *icaD* gene positive *S. aureus* isolates, the same authors explained with the possibility of mutation in the *icaA* gene. Using the sequencing analysis, Murugan et al. [27] have found a high similarity, but some differences in the *icaA* genes sequences of the 24 *S. aureus* isolates from conjunctivitis patients. Szweda et al. [28] pointed out that some mutations in *ica* genes are possible and this polymorphism may be the reason for the failed amplification of these genes. Considering that used positive control for *icaA* gene and 25% investigated isolates were positive during our investigation, we are of the same opinion that the mutations of *icaA* gene might be the reason for the negative results in our study. Therefore, we speculate that the presence of the *icaD* gene alone might indicate the existence of the *ica* locus in a total of 65.9% investigated *S. aureus* mastitis isolates. Giving that investigated isolates originated from different localities of the Republic of Serbia, the importance of these results is greater.

Distribution of the investigated *S. aureus* isolates is shown in Figure 3. Biofilm-related genes (both *icaA* and *icaD* genes, or *icaD* gene alone) were detected in *S. aureus* isolates originated from 7 out of 10 examined localities. Any potential biofilm-producer strains were not detected in Brasina, Mionica and Paraćin. Both *icaA* and *icaD* genes were not

Figure 3. Distribution of the investigated *S. aureus* mastitis isolates

found in all *S. aureus* isolates originated from the same herd (farms located in Stara Pazova, Padinska Skela and Petrovac). This fact indicates the existence of different *S. aureus* strains in the same herd. The tested genes (both *icaA* and *icaD* genes, or *icaD* gene alone) were also detected in *S. aureus* isolates originated from rural households in the municipalities of Čačak, Kraljevo, Tutin and Žagubica. Therefore, results of the present study indicate the existence of potential biofilm-producer strains, both under intensive and semi-extensive cows breeding.

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Authors' contributions

BS and VK defined the research theme, gave conception of the research and prepared manuscript. BS and JĐ did sample collections and laboratory studies. BS carried out the molecular studies. VK, VT, VD, NK and MD participated in the design of the research as well as in the analysis and interpretation of results. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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DETEKCIJA *icaA* I *icaD* GENA KOD IZOLATA *Staphylococcus aureus* IZOLOVANIH U SLUČAJU MASTITISA KRAVA U REPUBLICI SRBIJI

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Kao uzročnik kontagioznih mastitisa, *Staphylococcus aureus* (*S. aureus*) izaziva ozbiljne zdravstvene i ekonomske probleme. Sposobnost *S. aureus* da stvara biofilm se ističe kao važan faktor virulencije za uspostavljanje perzistentnih infekcija mlečne žlezde. Stoga je istraživanje imalo za cilj da ispita prisustvo *icaA* i *icaD* gena kod izolata *S. aureus* izolovanih u slučaju mastitisa krava, kao gena od značaja za formiranje biofilma. U cilju izolacije i identifikacije *S. aureus*, prikupljeno je ukupno 1555 uzoraka mleka poreklom od 401 krave iz različitih regiona Republike Srbije. Primenom konvencionalnih mikrobioloških metoda, 100 izolata je okarakterisano kao koagulaza pozitivne stafilokoke. Nakon primarne biohemijske identifikacije, izvršena je potvrda vrste 44 izolata *S. aureus* primenom MALDI-TOF masene spektrometrije, kao i PCR tehnike utvrđivanjem prisutva *nuc* gena koji je specifičan za vrstu *S. aureus*. Od svih ispitanih izolata *S. aureus*, 25,0% je posedovalo *icaA* i *icaD* gen. Prisustvo samo *icaD* gena je utvrđeno kod 40,9% izolata, tako da je ukupno 65,9% izolata bilo pozitivno na *icaD* gen. Preostalih 34,1% izolata *S. aureus* nije posedovalo ni *icaA*, ni *icaD* gen. Rezultati

ove studije ukazuju na prisustvo potencijalnih biofilm produkujućih sojeva *S. aureus* u različitim regionima Republike Srbije, kako u intenzivnom tako i u poluekstenzivnom uzgoju krava.